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Selective Antitumor Activity of MKT-077, a Delocalized Lipophilic Cation, on Normal Cells and Cancer Cells In Vitro

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Background and Objectives: 1-Ethyl-2-{[3-ethyl-5-(3-methylbenzothiazolin-2-yliden)]-4-oxothiazolidin-2-ylidenemethyl}pyridium chloride (MKT-077, formerly known as FJ776), a delocalized lipophilic cation, is known to accumulate in the mitochondria, according to the negative potential inside the mitochondria, and exert its cytotoxicity.
Methods: The single-cell suspensions of human cancer cell lines, human spleen cells, and fresh cancer specimens obtained from patients with gastric carcinoma were used for the 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H tetrazolium bromide (MTT) assay.
Results: The antitumor activity of MKT-077 was dose and concentration related, and 50% inhibitory concentrations (IC_{50}) ranged from 1.7 to 14.3 μg/ml, with a mean ± standard deviation (SD) of 8.4 ± 4.6 μg/ml. The IC_{50} of fresh surgical spleen-cell specimens ranged from 0.34 μg/ml to >100 μg/ml in a 48 h incubation, with a mean ± SD of 66.5 ± 37.7 μg/ml. When the antitumor activity of MKT-077 was compared between gastric cancer cells and spleen cells obtained from the same patient, the concentration-dependent antitumor activity of this agent was obvious in the cancer cells, while no significant cytotoxicity was observed in the spleen cells. The fresh surgical specimens of gastric cancer showed higher sensitivity to MKT-077 than did spleen cells at a concentration of 30 μg/ml, with a statistically significant difference at $P < 0.05$.
Conclusions: The selective antitumor activity of MKT-077 was confirmed using fresh surgical specimens and warrants further investigation.
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KEY WORDS: MKT-077; cancer cell lines; MTT assay; human spleen cells; human gastric cancer

INTRODUCTION

Delocalized lipophilic cations (DLCs) are known to accumulate in the mitochondria of cells, as demonstrated by the negative electrical potential inside the mitochondria [1–3]. Since carcinoma cells, particularly those of human origin, display an increased mitochondrial membrane potential when compared with normal epithelial cells, DLCs have been proposed as potential anticancer agents [4–26]. A DLC with antitumor properties, 1-ethyl-2-{[3-ethyl-5-(3-methylbenzothiazolin-2-yliden)]-4-oxothiazolidin-2-ylidenemethyl}pyridium chloride (MKT-077, formerly known as FJ776), was initially synthesized at Fuji Photo Film Co. (Ashigara, Kanagawa, Japan). Previous reports characterized its accumulation in mitochondria and its cytotoxicity against cancer cells

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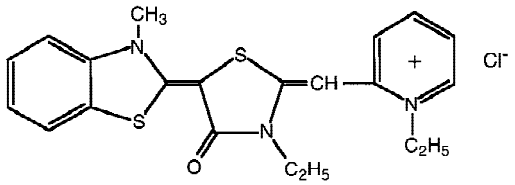


Fig. 1. Molecular structure of MKT-077 (formerly known as FJ776; $C_{12}H_{22}N_3OS_2Cl$, molecular weight = 432.01).

from rodents and humans [27–29]. In the present study, we further investigated the cytotoxicity of MKT-077 using cultured human cancer cells, as well as human normal cells (spleen) and tumor cells obtained from fresh surgical specimens.

MATERIALS AND METHODS

Agent

MKT-077 (formerly known as FJ776; $C_{12}H_{22}N_3OS_2Cl$, molecular weight = 432.01) was chemically synthesized at Fuji Photo Film Co. The structure (Fig. 1) was determined by X-ray analysis of its crystal and various spectroscopic data (e.g., ^{1}H-nuclear magnetic resonance, ^{13}C-nuclear magnetic resonance, infrared, and mass). MKT-077 is orange in color (maximum absorbance at 495 nm) and highly soluble in water (>200 mg/ml) [29].

Cultured Human Cancer Cells and Fresh Surgical Specimens

We used the following human tumor cell lines: MKN45 and MKN74 (gastric cancer); Colo 205, WiDr, C-1, HT-29, and LS174T (colon cancer); and CRL 1420 (pancreatic cancer). MKN45 and MKN74 were purchased from Dai Nippon Pharmaceutical Co., Ltd. (Tokyo, Japan). Colo 205 and WiDr were provided by Roche Research Institute (Kamakura, Japan). C-1 and HT-29 were provided by the Pathology Division, National Cancer Center Research Institute (Tokyo, Japan). LS 174T and CRL 1420 were obtained from American Type Culture Collection (Bethesda, MD). All cell lines were grown in RPMI-1640 medium (Life Technologies, Inc., Gaithersburg, MD) containing 10% w/v fetal calf serum, 100 IU penicillin, 100 μg streptomycin, and 0.25 μg/ml amphotericin B (conditioning medium).

Surgical specimens were obtained from 27 patients with stage III or stage IV gastric cancer. All patients gave informed consent. When splenectomy was performed at the same time as the total gastrectomy, the spleens of 10 patients were also used for the experiments.

Cell Preparation

After surgery, the resected specimen was stored in Hanks' balanced salt solution containing 100 IU penicillin, 100 μg streptomycin, and 0.25 μg/ml amphotericin B (Hanks' solution), and brought to the laboratory as soon as possible. Single-cell suspensions were prepared enzymatically for 30 min using 0.5 mg/ml pronase, 0.2 mg/ml collagenase type I, and 0.2 mg/ml DNase suspended in Hanks' solution. After two centrifugations, tumor cells were suspended in the conditioning medium and diluted to a concentration between 2×10^5 and 1×10^6 cells/ml.

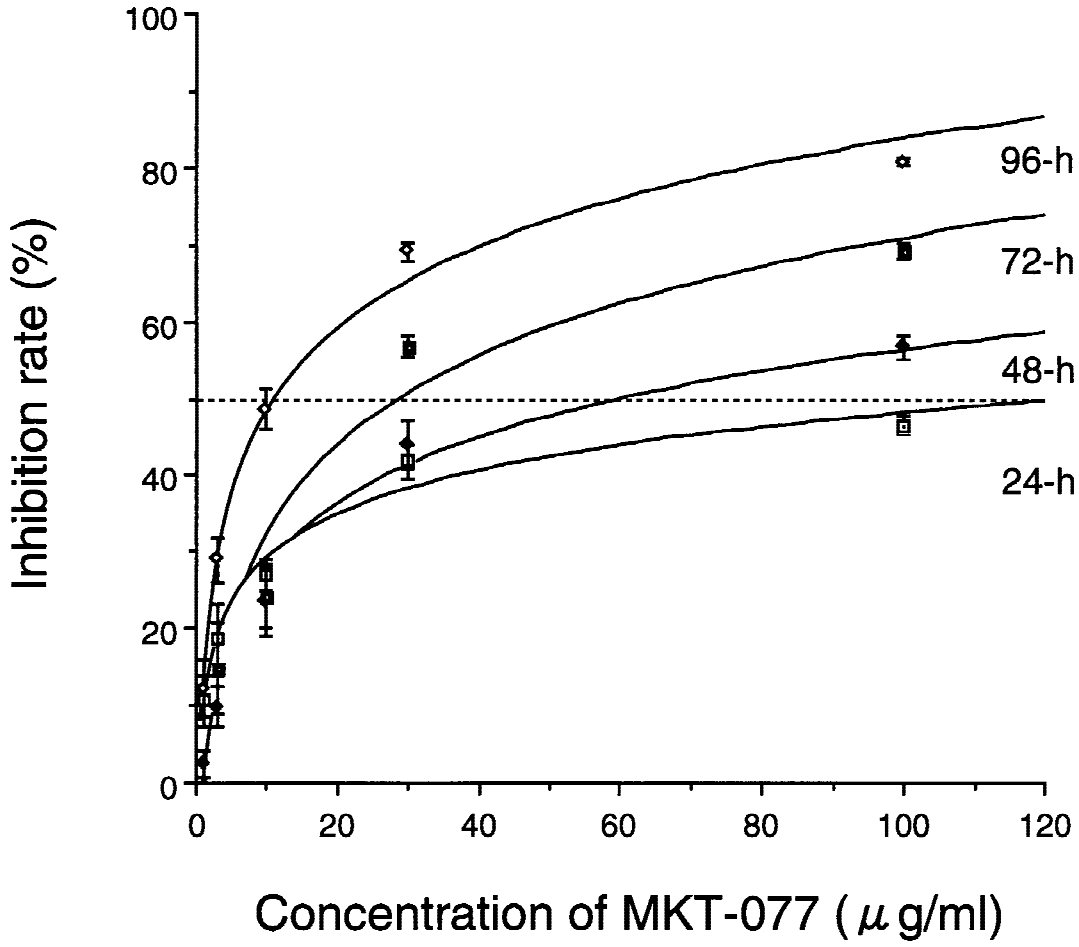
In cases where splenectomy was performed with total gastrectomy, a part of the resected spleen was also stored in Hanks' solution and used in the chemosensitivity assay with MKT-077. Scissors were used to cut a portion of the red pulp of the spleen into $3 \times 3 \times 3$ mm pieces. These pieces were then suspended in the conditioning medium at a concentration of approximately 1×10^8 cells/ml. The cell suspension was plated into 96-well microplates (GIBCO, Gaithersburg, MD) in volumes of 50 ml at 10^3–10^4 cells/well for cancer cell lines and 10^4–10^5 cells/well for fresh surgical specimens of cancerous cells and spleen cells.

Evaluation of Antitumor Activity

The 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyl-2H tetrazolium bromide (MTT; Sigma Chemical Co., St. Louis, MO) assay, reported by Mosmann [30] with some modifications [31–34], was used in evaluating the *in vitro* chemosensitivity of the cancer cell lines, fresh surgical specimens, and spleen cells.

The medium containing 0.3, 3, 10, 30, and 100 μg/ml of MKT-077 or control was incubated for 24, 48, 72, and 96 h at 37°C in a humidified atmosphere of 95% air and 5% CO_2. At the end of incubation, the media were exchanged again to the conditioning medium after centrifugation, and the cells were incubated for a further 48 h. After this recovery incubation period, we added a mixture of MTT at 4 mg/ml and sodium succinate at 0.1 M dissolved in phosphate-buffered saline that had been filtered through a 0.45 mm membrane filter (Millipore, Bedford, MA). The plate was incubated for 4 h at 37°C. After the final incubation, 150 μl/well dimethyl sulfoxide was added to dissolve the MTT-formazan product. The plate was mechanically shaken for a few minutes on a mixer (Model 250, Sonifier, Branson, MO) to dissolve the formazan salt. The optical density of the solution at a volume of 150 μl/well was read on model EAR 340 Easy Reader (SLT-Lab Instruments, Salzburg, Austria) at 570–630 nm. The inhibition rate was calculated using the formula: $(1 - A/B) \times 100\%$, where A and B represent the mean absorbance of the treated and control wells, respectively. The 50% inhibitory concentration (IC_{50}) in μg/ml was calculated from the linear regression equation of concentration vs. inhibition rate.

Most of the surgical specimens of gastric cancer were tested at 30 μg/ml of MKT-07 because the number of specimens was insufficient for the evaluation across several concentrations.



Fgi. 2. Concentration- and time-dependent antitumor activity of MKT-077 against C-1, a human colon cancer cell line. Tumor cells were exposed to various concentrations of MKT-077 for specified time periods. Cell viability was detected by MTT assay. The data were indicated as mean ± standard error in mg/ml.

Statistical Analysis

All of the statistical analyses were performed using the Student *t* test, with $P < 0.05$ regarded as statistically significant. *P*-values were determined using results obtained from one-tailed tests.

RESULTS

The cytotoxicity of MKT-077 against C-1, a human colon cancer cell line, was positively correlated with time of exposure from 24 to 96 h and with concentration (Fig. 2). When IC_{50} was plotted against contact time, the relationship was linear with a regression equation of $IC_{50} = 154.08 - 1.5 \times$ (contact time in h), suggesting that the antitumor activity of MKT-077 depends on the area under the concentration × time curve *in vitro* (Fig. 3). On the basis of these results, the incubation time of the cultured human cancer cell lines was fixed as 72 h in the subsequent experiments. Table I shows the IC_{50}s of these cell lines, which ranged from 1.7 to 14.3 μg/ml, with a mean ± standard deviation (SD) of 8.4 ± 4.6 μg/ml.

The antitumor activity of MKT-077 on spleen cells is shown in Table II. The IC_{50}s were all greater than 100 μg/ml in case numbers 2, 4, 7, 8, and 10, although a concentration-dependent antitumor activity was observed from 1 to 100 μg/ml. These IC_{50}s were described as $>10^2$ μg/ml in Table II and valued at 100 μg/ml in the statistical analyses. As a result, IC_{50} ranged from 0.34 to >100 μg/ml, with a mean ± SD of 66.5 ± 37.7 μg/ml. Figure 4 shows the cytotoxicity of MKT-077 against gastric cancer cells and normal spleen cells obtained from the same patient. While the concentration-dependent antitumor activity of this agent was obvious in the cancer cells, no significant cytotoxicity was observed in the normal cells. This indicates a selective anticancer activity of MKT-077 on cells originating from the same host. The differing chemosensitivities of fresh surgical specimens of gastric cancer cells and normal spleen cells are shown in Figure 5. When the inhibition rate was plotted at a

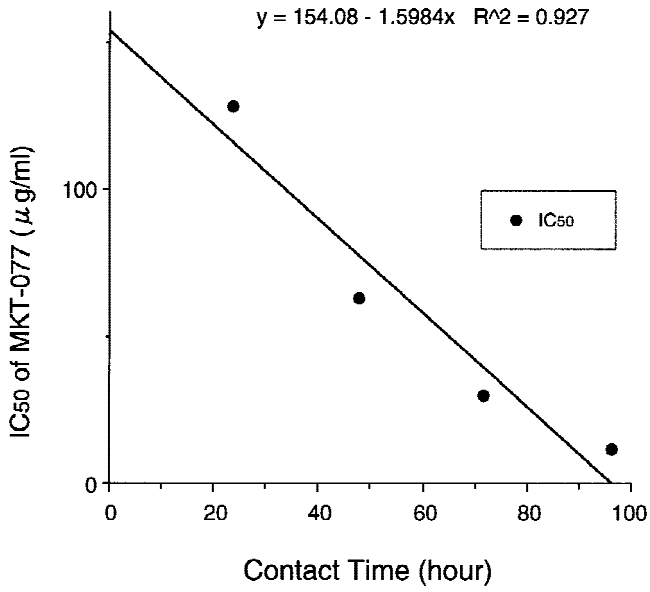


Fig. 3. Correlation between IC_{50} of MKT-077 and contact time on C-1 cells. The linear correlation suggests that the antitumor activity of MKT-077 is dependent on the area under the concentration × time curve.

TABLE I. Antitumor Activity of MKT-077 on Cultured Human Cancer Cell Lines*

Original organ	Cell line	IC_{50} (μg/ml)
Gastric cancer	MKN74	11.5
	MKN45	9.1
Colon cancer	Colo 205	3.5
	WiDr	11.1
	C-1	4.2
	HT-29	1.7
	LS174T	11.6
Pancreatic cancer	CRL 1420	14.3
Mean ± SD		8.4 ± 4.6

*Cell lines were exposed to various concentrations of MKT-077 continuously for 72 h. Cell viability was detected by MTT assay.

concentration of 30 μg/ml, the inhibition rates were 50.3 ± 17.7% for gastric cancer cells and 35.6 ± 17.3% for spleen cells, with a statistically significant difference at $P < 0.05$.

DISCUSSION

To identify a suitable antitumor agent, 200,000 DLCs were initially screened. Of these, 100,000 compounds were further evaluated at Fuji Photo Film Co. [29]. From these candidates, 1,000 rhodacyanines were synthesized and characterized as to solubility, stability, pharmacokinetics, and antitumor activity on cultured cell lines [14,29]. Of these samples, MKT-077 was chosen for further evaluation as an antitumor agent because it is highly soluble in water and has selective cytotoxicity toward cancer cells compared with normal cells. The selective

TABLE II. Antitumor Activity of MKT-077 on Fresh Surgical Specimens of Human Spleen Cells*

Case No.	IC_{50} (μg/ml)	IR_{30}[a]
1	49.6	35.0
2	$>10^2$	0.16
3	31.9	48.0
4	$>10^2$	23.0
5	0.34	63.0
6	44.9	45.0
7	$>10^2$	33.0
8	$>10^2$	22.8
9	37.8	44.0
10	$>10^2$	42.0
Mean ± SD	66.5 ± 37.7	35.6 ± 17.3

*Cell lines were exposed to various concentrations of MKT-077 continuously for 48 h. Cell viability was detected by MTT assay.
[a]Inhibition rate at 30 μg/ml.

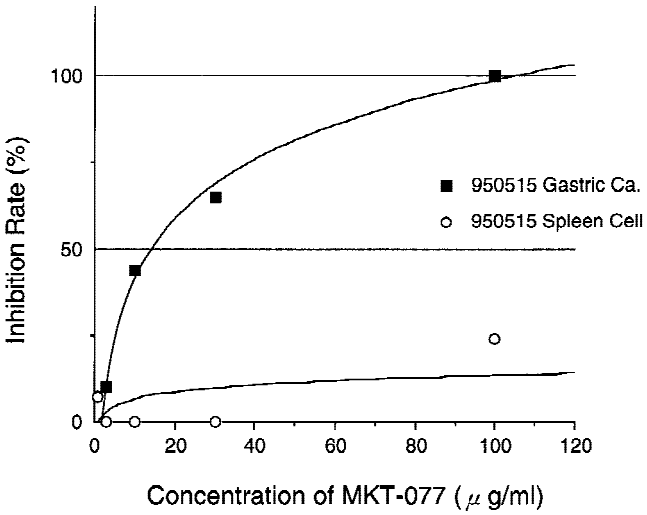


Fig. 4. Selective antitumor activity of MKT-077 on human gastric cancer and spleen cells obtained from the same host. While the concentration-dependent antitumor activity of this agent was obvious in the cancer cells, no significant cytotoxicity was observed in the normal spleen cells. This indicates a selective antitumor activity of MKT-077 on cells originating from the same host.

toxicity of this compound had already been confirmed using human cancer cell lines and the monkey kidney CV-1 cell line, and its in vivo cytotoxicity had been suggested using human tumor xenografts in nude mice [29]. The selective antitumor activity of MKT-077 was explained by its selective uptake by cancer cells (i.e., 65-fold greater than by CV-1 cells), resulting in inhibition of respiratory and electron transport activity in the mitochondrial membrane [27–29].

The present study is concerned with the selective cytotoxicity of MKT-077 using cultured human cancer cell lines and fresh surgical specimens of gastric cancer cells and spleen cells that were resected from the same patient. The antitumor activity of MKT-077 on the C-1 cell line

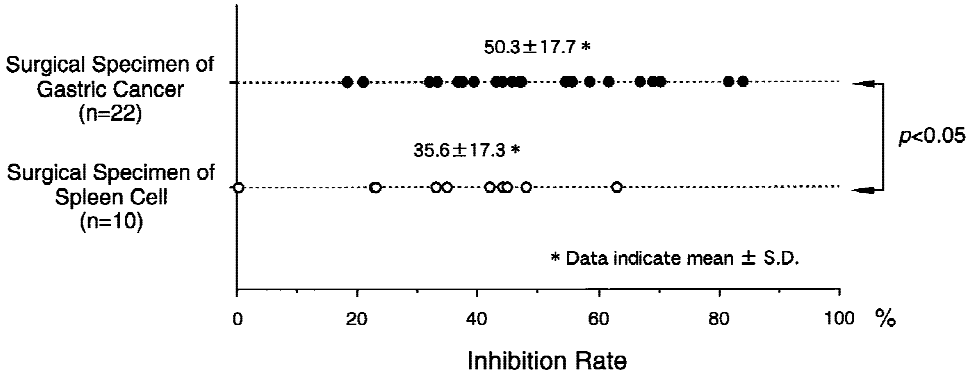


Fig. 5. Chemosensitivity of fresh surgical specimens of gastric cancer cells and normal spleen cells exposed to MKT-077. Cells were exposed to MKT-077 continuously for 48 h at a concentration of 30 μg/ml. Cell viability was detected by MTT assay. The inhibition rates were 50.3 ± 17.7% for gastric cancer cells and 35.6 ± 17.3% for spleen cells, with a statistically significant difference at $P < 0.05$.

was positively correlated with duration of exposure and with concentration. Thus, antitumor activity was dependent on the area under the time × concentration curve. Given that a 72 h incubation had sufficient antitumor activity against C-1 cell lines, further experiments using MKT-077 concentrations from 0.3 to 100 μg/ml used a 72 h length of contact. In these experiments, the IC_{50} ranged from 1.7 μg/ml for HT-29 to 14.3 μg/ml for CRL 1420, with a mean ± SD of 8.4 ± 4.6 μg/ml. The sensitivity of gastric cancer cell lines was almost equivalent to that of the colon cancer cell lines without the specific antitumor spectrum of this compound.

When fresh surgical specimens of human spleen cells were used for the chemosensitivity assay of MKT-077, the cells were incubated for only 48 h because the viability of control spleen cells diminishes after 72 h. The IC_{50}s of spleen cells ranged from 0.34 to >100 μg/ml, with a mean ± SD of 66.5 ± 37.7 μg/ml. It is noteworthy that the IC_{50}s of 5 of 10 specimens were >100 μg/ml. When the upper concentration limit of MKT-077 was set at 30 μg/ml, all of the spleen cells were determined to be insensitive to MKT-007 as assessed by an MTT assay with a 48 h incubation. Moreover, the fresh surgical specimens from gastric cancer showed higher sensitivity to MKT-077 than spleen cells even though both were obtained from the same host. Since the number of fresh surgical specimens of gastric cancer was limited, the assay of the cancerous tissue was conducted using a cutoff concentration of 30 μg/ml. The inhibition rates of gastric cancer cells ranged from 18% to 84%, with a mean ± standard deviation of 50.3 ± 17.7%, which was higher than those of normal spleen cells ($P < 0.05$). A selective antitumor activity of MKT-077 on fresh surgical specimens of gastric cancer cells compared with spleen cells was demonstrated. Since the sensitivity of these spleen cells is representative of the sensitivity of normal lymphoid cells, one might expect the cytotoxic side effects of MKT-007 in bone marrow suppression to be minimal. These results suggest that MKT-077 selectively kills malignant cells and warrants further investigation.

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